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The endogenous langur type D retrovirus PO-1-Lu and its exogenous counterparts in macaque and langur monkeys

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Abstract

PO-1-Lu, the endogenous type D retrovirus of langurs (*Trachypithecus obscurus*) has previously been considered a progenitor to the prototype type D retrovirus, Mason Pfizer monkey virus (M-PMV/SRV-3), that became established in macaque monkeys (*Macaca* spp.) following a zoonosis. This study reevaluates this hypothesis to include other exogenous SRVs. New sequence information from the gp70(SU)-encoding region of PO-1-Lu shows striking similarity to the newly identified exogenous langur retrovirus, SRV-6, recently isolated from the Hanuman Langur (*Semnopithecus entellus*). An unrooted, bootstrapped neighbor-joining tree derived from *env* gene nucleotide sequences shows PO-1-Lu and SRV-6 appear more closely related genetically to SRV-2 than SRV-1 or SRV-3 (M-PMV). This is also reflected in our observations that the M-PMV envelope glycoprotein precursor $gPr86^{Env}$ and gp70(SU) were antigenically distinct from PO-1-Lu, although the gp22(TM) glycoproteins were antigenically cross-reactive. The potential that SRV-6 represents an exogenous form of PO-1-Lu that has arisen following a recent zoonosis is discussed.

Keywords: PO-1-Lu; Simian retrovirus; Zoonosis; Exogenous type D retrovirus; Endogenous type D retrovirus; Macaque monkeys; Langur monkeys; Evolution

Introduction

The langur endogenous retrovirus PO-1-Lu derives its name from its species of isolation, *Prebytis obscurus* (PO), the Spectacled Langur, now classified as *Trachypithecus obscurus*, the Dusky Leaf-monkey. PO-1-Lu was originally isolated in 1977 following cocultivation of iododeoxyuridine-treated langur lung tissue with bat lung cells (Tb-1-Lu) and acquired the name PO-1-Lu (Todaro et al., 1978). PO-1-Lu was subsequently found to be antigenically and genetically related to the exogenous prototype type D retrovirus, Mason Pfizer monkey virus (M-PMV), first isolated in 1970 from an 8-year-old rhesus macaque (*Macaca mulatta*) (Chopra and Mason, 1970; Jensen et al., 1970). This led to the hypothesis that PO-1-Lu could represent a progenitor to M-PMV (Benveniste and Todaro, 1977).

During the 1980s several exogenous type D retroviruses were isolated from diverse macaque species at primate centers in the United States from imported monkeys. These were eventually grouped according to their neutralization specificity and a nomenclature adopted, namely, simian retrovirus (SRV) serotypes 1–5. M-PMV is analogous to SRV-3. The exogenous type D retroviruses, SRV-1, -2, and -3, are associated with a horizontally transmitted simian immunodeficiency disease, simian AIDS-D (SAIDS-D), of varying severity and exclusively in macaque monkeys (*Macaca* spp.) (Gardner and Marx, 1985). SRV-2 is also associated with retroperitoneal fibromatosis and has been used as an animal model to study human Kaposi's sarcoma (Marx et al., 1985). SAIDS-D is distinct from acquired immunodeficiency syndrome (AIDS) associated with the human and simian immunodeficiency viruses HIV/SIV

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(Bryant et al., 1985). A new member of the exogenous type D retroviruses, provisionally named SRV-6, has recently been isolated from an apparently healthy wild caught Hanuman Langur (*Semnopithecus entellus*), previously classified as *Presbytis entellus*, in India (Nandi et al., 2000). It is presently less well characterized and its antigenic relationship to SRV1-5 and neutralization specificity remains to be determined.

Early work showed that PO-1-Lu and M-PMV shared some profound similarities. The products of the *gag* gene for M-PMV and PO-1-Lu were found to be of similar size (Colcher et al., 1978). Furthermore, the major capsid proteins were antigenically cross-reactive because they competed with one another in interspecies competitive radioimmunoassays (Colcher et al., 1977; Bryant et al., 1978) and produced similar patterns on two-dimensional gel electrophoresis following trypsin digestion. The genomic relatedness of PO-1-Lu and M-PMV was determined by mutual hybridization of [^{3}H] cDNA for each virus with M-PMV and PO-1-Lu viral RNA (Bryant et al., 1978). PO-1-Lu and M-PMV, however, exhibited certain differences; for example, PO-1-Lu had a narrower host range compared to M-PMV, infecting only bat lung and human cells (Todaro et al., 1978). PO-1-Lu and M-PMV nevertheless exhibited reciprocal receptor interference on human cells indicative of shared receptor utilization (Sommerfelt and Weiss, 1990). These viruses currently constitute two members of the largest receptor interference group on human cells to date that includes primate type D retroviruses, certain type C retroviruses (reviewed in Sommerfelt, 1999), and the human endogenous virus HERV-W (Blond et al., 2000). The receptor was recently identified as a sodium-dependent transporter encoded by the hATB^0 gene (Rasko et al., 1999; Tailor et al., 1999).

The exogenous viruses SRV-1, -2, and M-PMV have been molecularly cloned and sequenced (Power et al., 1986; Sonigo et al., 1986; Thayer et al., 1987). They show significant sequence similarity suggesting that they have a common progenitor. No sequence information is available for SRV-4, SRV-5, or PO-1-Lu. M-PMV has a genomic size of 8.1 kb that encodes four genes in the order 5′-*gag-pro-pol-env*-3′. The *gag* gene encodes the structural proteins that are expressed from a polyprotein precursor Pr78^{Gag} in the order NH_2-p10(MA)-pp18/24-p12-p27(CA)-p14(NC)-p4-COOH (Sonigo et al., 1986). The envelope gene is synthesized as a polyprotein precursor gPr86^{Env} from a singly spliced messenger RNA (mRNA). The envelope precursor is cleaved by a cellular endopeptidase in a late Golgi compartment generating the surface glycoprotein gp70(SU) that interacts with available receptors on susceptible cells and the transmembrane glycoprotein gp22(TM) that anchors the envelope glycoprotein in the viral and plasma membrane and is also required during entry for membrane fusion. On viral maturation gp22(TM) is cleaved by the viral protease to gp20(TM) (Brody et al., 1992).

The observed similarities between PO-1-Lu and M-PMV, coupled with the observation that M-PMV [^{3}H]cDNA hybridized better to langur DNA than colobus DNA, suggested that M-PMV was likely derived from one of the Asian genera of the primate subfamily Colobinae, perhaps the langur monkeys themselves (Benveniste and Todaro, 1977). This was an attractive hypothesis because both macaque and langur monkeys inhabit overlapping geographical locations in India and South East Asia. The natural prevalence of the exogenous SRVs including M-PMV in feral macaque monkeys in India and South East Asia is currently not clear. These viruses are presumably present in feral simians since infected monkeys have been imported to primate centers in the United States in the past.

In this study the mutual hybridization specificity between the genomes of M-PMV and PO-1-Lu was used to obtain a genomic size for PO-1-Lu proviral DNA and to determine restriction endonuclease patterns, which were compared to that of M-PMV. Oligonucleotide primer sequences for polymerase chain reaction (PCR) were designed to amplify a region of the PO-1-Lu envelope gene equivalent to that recently identified for SRV-6. DNA sequencing of this product allowed a comparison of the nucleotide sequence of this region of the PO-1-Lu genome to M-PMV and other retroviruses with related envelope genes. This genetic information was then compared to our observations on the antigenic cross-reactivity of M-PMV and PO-1-Lu envelope glycoprotein precursor formation and processing. Our results strengthen the likelihood that PO-1-Lu represents a progenitor to M-PMV and the exogenous type D retroviruses and that SRV-6 probably represents its closest exogenous relative.

Results

PO-1-Lu genomic size and restriction endonuclease patterns

Proviral DNA for both M-PMV and PO-1-Lu was extracted by the method of Hirt (1967). This resulted in the isolation of predominantly linear forms of both M-PMV and PO-1-Lu proviral DNA. Using labeled M-PMV proviral DNA as a probe, a band of 8.1 kb was identified for M-PMV and a band of 8.4 kb for PO-1-Lu following gel electrophoresis. Restriction endonuclease digestion of M-PMV and PO-1-Lu proviral DNA with enzymes *Nco*I, *Nde*I, and *Pst*I, resulted in distinct fragment patterns for M-PMV and PO-1-Lu respectively for each enzyme (Fig. 1). This shows that although these viruses are genetically related due to mutual hybridization, they are distinct in overall proviral DNA sequence.

DNA sequence information for PO-1-Lu shows greatest similarity to the exogenous langur retrovirus SRV-6

A novel type D retrovirus was recently isolated from the Hanuman langur, *S. entellus* (Nandi et al., 2000). To carry

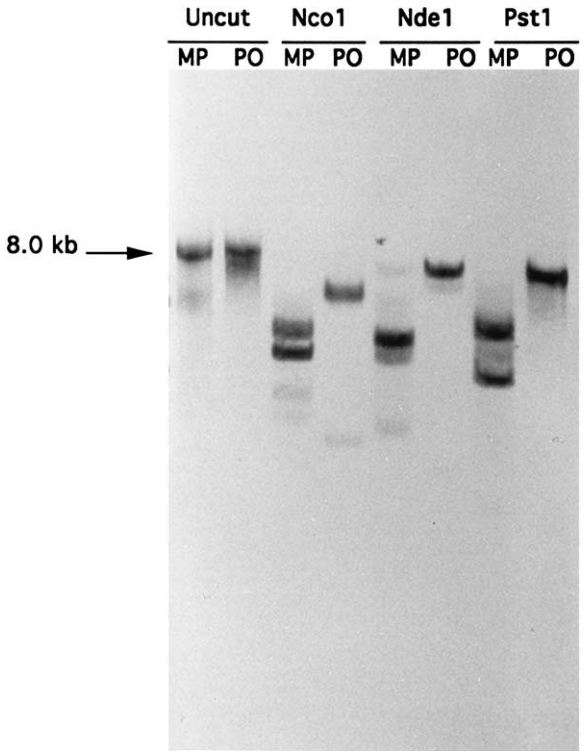

Fig. 1. Gel electrophoresis of Hirt extracts from M-PMV and PO-1-Lu infected cell cultures, and subsequent restriction endonuclease digestion with *Nco*I, *Nde*I, and *Pst*I.

out successful PCR amplification, the authors used primers designed for SRV-2 (Grant et al., 1995). In our hands, these primers did not amplify PO-1-Lu DNA, similarly, primers based on SRV1-3 genetic sequences did not amplify PO-1-Lu genes (data not shown). Using the sequence information available for SRV-6 and SRV-2, new primers were designed that could successfully amplify a region of PO-1-Lu *env* from HOS-infected cell lysates. The resulting product was approximately 270 nucleotides in length when measured by gel electrophoresis on a 2% agarose gel. On sequencing in both forward and reverse orientations, unequivocal sequence information for 266 nucleotides was obtained. This sequence was translated to yield a sequence of 88 amino acids in length that was then aligned to the corresponding regions of the envelope glycoproteins from 17 retroviruses (Fig. 2). Interestingly, the PO-1-Lu sequence showed greatest homology to SRV-6 where only 12 amino acid residues were different. This region of the PO-1-Lu gp70 also showed significant similarity in amino-acid sequence to that of SRV-2 and SRV-3/M-PMV. Furthermore, certain amino acid clusters were conserved between PO-1-Lu and the SRV-1, -2, and -3/M-PMV sequences analyzed. These include the amino motifs “SDGGGPQD” “YHPLALP”, “RGKEKIDA”, and “CWL”.

The amplified PO-1-Lu DNA sequence was then subjected to tree analysis and aligned to the entire envelope genes of the viruses shown in Fig. 2 using the Clustal X(1.8) program. An unrooted bootstrapped neighbor-joining tree was derived from this alignment and is shown in Fig. 3. The laboratory strain HIV_HXB2 was used as an outgroup. Fig. 3, based on the limited sequence information we have obtained, shows that PO-1-Lu clusters with SRV-6. PO-1-Lu and SRV-6 are also found to group more closely to SRV-2 sequences than SRV-1 and SRV-3, while the endogenous RD114, BaEV, and SERV sequences form a distinct group on a separate node from the exogenous type D retroviruses and PO-1-Lu. The endogenous viruses of the new world monkey SMRV-H, and the recently identified endogenous virus from a marsupial (TvERV/D), as well as avian reticuloendotheliosis virus (REV) and spleen necrosis virus (SNV) group together. HERV-W was clearly most divergent.

```
PO-1-Lu             GQPVCWNSRP PLHISDGGGP QDKAREIMVH KKLEELQKSL FPELHYHPLA LPKARGKEKI DAQTFDLLTV THSLLNNS.N SDLANDCWL
AF187057_SRV-6      -------TH- ---T------ --RV------ ---------- Y--IR----- ---I------ ---------A --N-----.- -S-------
L38695_SRV-2        --HI---PKA -V-------- -------A-Q -R---IHR-- ----R----- ---------- -----N---A -Y----K-.- PN---E---
AF126467_SRV-2      --HI---PKA -V-------- -------A-Q -R---IHR-- ----R----- ---------- -----N---A -Y----K-.- PN---E---
AF126468_SRV-2      ---I---PKA -V-------- -------A-Q -R---IHR-- ----R----- ---------- -----N---A -Y----K-.- PN---E---
M16605_SRV-2        ---I---PKA -V-------- -------A-Q -R---IH--- ----R----- ---------- -----N---A -Y----K-.- PN---E---
M12349_MPMV/6A      --V------- SV-------- -----D-I-N --F---HR-- ----S----- --E------- --H-L---AT V-----A-.Q PS--E-----
AF033815_SRV-3      --V------- SV-------- -----D-I-N --F---HR-- ----S----- --E------- --H-L---AT V-----A-.Q PS--E-----
M11841_SRV-1        --V-----Q- SV-M------ ---V---I-N --F---H--- ----S----- --E------- --H-----AT V-----V-SQ RQ--E-----
D10032_BaEV/M7      --SI--STTA -I-V------ L-TT-IKS-Q R----IH-A- Y---Q----- I--V-DNLMV ----LNI-NA -YN--LM-.- TS-VD----
U85506_SERV         ------STTA -I-------- L-TT-IKT-Q -----IH-A- Y--IQ----- ---V-DNLM- ------I-NA -YN--QM-.- TS--H-----
U85505_SERV         -K----STTA -I-------- L-T--IKT-Q R----IH-A- Y---Q----- --EI-DNFRL ------I-NA -*
X87829_RD114        N-----SATA -I-------- L-TK-VWT-Q -R--QIH-AM T---Q----- ---V-DDLSL --R---I-NT -FR--QM-.- FS--Q-----
M23385_SMRV-H       -KS----QQA -I-V------ --AV--LY-Q -QI-LVIQ-Q --K-S----- RS-P--PD.- ---ML-I-SA --QA--I-.- PS--QN---
X01455_REV          WETSMLGP-C -CVCL----- T-RFG.RICA EG---IIRHS Y-SVQ----- --RP--VD.L -P--S-I-EA --QV--AT.- PQ--EN---
M87666_SNV          -K----DPVA -VYV------ T-MI--ES-R ER---IIRHS Y-SVQ----- --RS--VD.L -P--S-I-EA --QV--AT.- PK--EN---
AF284693_TvERV/D    -KN---SLHA -I-V------ T-QI--ME-K ERVD-IIRAM Y-P-Q----- --RG--VD.L -V--Y-I-AS --KA--IT.- P---K----
AF072506_HERV-W     GVT---TYFT QTGM-----V --Q---KH-K EVISQ-TRVH GTSSP-KG-D -S-LHETLRT HTRLVS-FNT -LTG-HEV.S AQNPTN--I
```

Fig. 2. Alignment of a PO-1-Lu amino acid sequence with the corresponding regions of related envelope glycoproteins. .indicates a gap in the sequence, *indicates a stop codon, −indicates an amino acids identical to PO-1-Lu. SRV-1-6, simian retroviruses serotypes 1-6; M-PMV/6A, Mason Pfizer monkey virus clone 6A; BaEV/M7, baboon endogenous retrovirus strain M7; SERV, simian endogenous retrovirus, RD114, cat endogenous retrovirus; SMRV-H, squirrel monkey retrovirus isolated from a human cell line; REV, reticuloendotheliosis virus; SNV, spleen necrosis virus; TvERV/D, *T. vulpecula* endogenous retrovirus type D; HERV-W, human endogenous retrovirus-W. Accession number to each viral DNA sequence is shown alongside each virus.

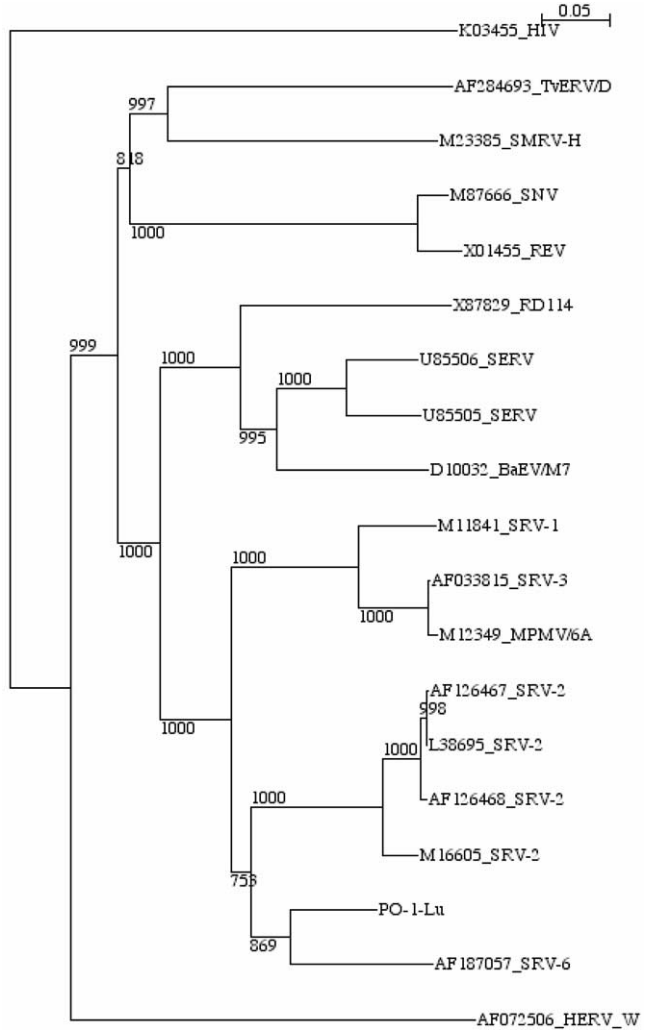

Fig. 3. An unrooted bootstrapped neighbour-joining free of the amplified DNA sequence of PO-1-Lu within gp70 and the entire envelope gene for retroviruses that are related or belong to the same receptor interference group on human cells (with the exception of SRV-6 where only an envelope gene fragment can be compared). SRV-1–6; simian retroviruses serotypes 1–6, M-PMV/6A, Mason Pfizer monkey virus clone 6A; BaEV/M7, baboon endogenous retrovirus strain M7; SERV, simian endogenous retrovirus, RD114, cat endogenous retrovirus, SMRV-H, squirrel monkey retrovirus isolated from a human cell line, REV, reticuloendotheliosis virus; SNV, spleen necrosis virus; TvERV/D, *T. vulpecula* endogenous retrovirus type D; HERV-W, human endogenous retrovirus-W. Accession number to each viral DNA sequence is shown alongside each virus.

Antigenic cross-reactivity between M-PMV and PO-1-Lu envelope glycoproteins

To determine the effects of sequence heterogeneity between M-PMV and PO-1-Lu on antigenic cross-reactivity, infected cell lysates were immunoprecipitated with a goat anti-MPMV polyclonal antibody. Fig. 4A shows that the M-PMV envelope polyprotein precursor $gPr86^{Env}$ is synthesized in the pulse (P) and subsequently processed to the surface glycoprotein gp70(SU) evident in the chase (C). The upper panel shows an overnight exposure, and the lower panel, a 14-day exposure of the gel. However, neither $gPr86^{Env}$ nor gp70(SU) were detectable for PO-1-Lu using this antibody. Fig. 4B shows pulse-chase immunoprecipitation using a mouse anti-gp22 monoclonal antibody raised against SRV-1. The M-PMV polyprotein precursor $Pr86^{Env}$ is clearly visualized in the pulse (P) and subsequently cleaved to gp22(TM) and gp20(TM) detectable in the chase (C). Only the virion-associated gp20(TM) is released into the medium (M). The anti-gp22(TM) antibody, however, failed to detect an envelope polyprotein precursor for PO-1-Lu in the pulse (P) although both PO-1-Lu gp22(TM) and gp20(TM) are clearly evident in the chase (C). Virion-associated gp20(TM) is faintly evident released into the medium (M). These results show that the gp22/20(TM) is antigenically cross-reactive between M-PMV and PO-1-Lu; however, the $gPr86^{Env}$ and gp70(SU) glycoproteins are antigenically distinct.

Discussion

In this study we have addressed both the genetic and antigenic relationship of PO-1-Lu to M-PMV. The mutual hybridization of proviral DNA between PO-1-Lu and M-PMV was exploited to obtain a genomic size for PO-1-Lu (8.4 kb). Despite mutual hybridization of linear proviral DNA, the M-PMV and PO-1-Lu genomes were not identical since they exhibited distinct restriction endonuclease patterns on digestion with *Nde*I, *Nco*I, and *Pst*I (Fig. 1). We cannot rule out the possibility at this time that certain other restriction endonucleases may have given rise to common fragments.

PCR primers based on the limited sequence information available for SRV-6 and sequence information available for SRV-2 amplified a short region of the PO-1-Lu envelope gene encoding gp70(SU). This DNA sequence showed remarkable similarity to that of SRV-6 (84% nucleic acid identity), which could not have been a result of contamination since we do not have access to SRV-6-infected cultures. On translation, the PO-1-Lu amino acid sequence retained a remarkable similarity (86% amino acid identify) to that of SRV-6 (Fig. 2). The amino acid sequence information provided by Nandi et al. (2000) is predicted from sequencing one DNA strand only and therefore the possibility for minor errors cannot be ruled out. The DNA sequence used to predict the amino acid sequence for PO-1-Lu in Fig. 2 has been confirmed in both forward and reverse orientations. Interestingly PO-1-Lu showed greater amino acid identity to SRV-2 strains (77–78%) than to M-PMV/SRV-3 (76%) or to SRV-1 (75%) within this region. It is possible that these viruses may exhibit a different pattern of identity for other regions of the genome (for example, Gag). The sequence heterogeneity between M-PMV and PO-1-Lu within this region of gp70(SU) was confirmed by looking at antigenic cross-reactivity between M-PMV and PO-1-Lu. Fig. 4A shows that the gp70(SU) of these viruses are antigenically

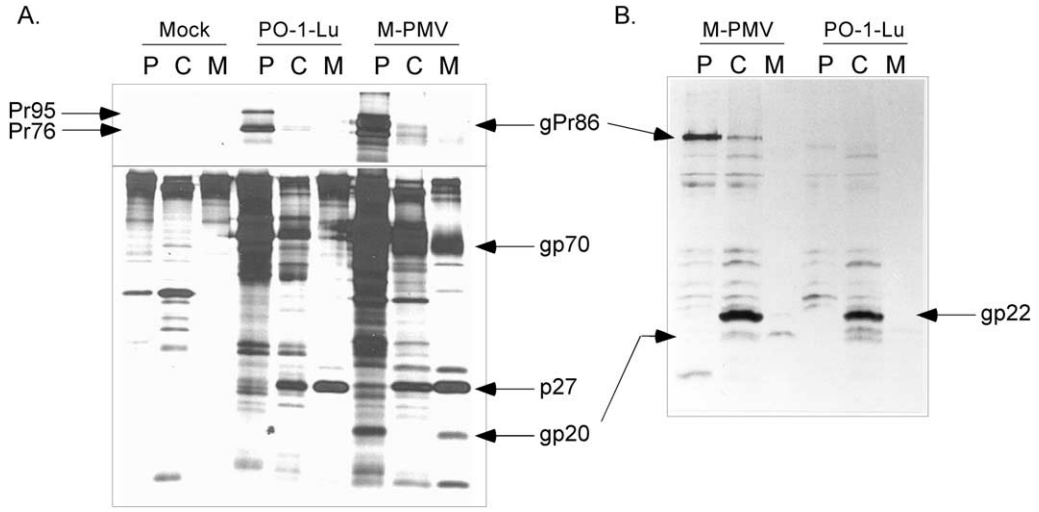

Fig. 4. Pulse-chase immunoprecipitation of M-PMV and PO-1-Lu from-infected cell cultures. Lanes P, C, and M refer to pulse, chase, and medium, respectively. (A) Immunoprecipitation using goat anti-MPMV polyclonal antibody. The upper panel shows overnight exposure of the polyprotein precursors for $Pr76^{Gag}$, $Pr95^{Gag\text{-}Bro}$, and $gPr86^{Env}$. Lower panel shows 14-day exposure of the same gel. Here, proteolytic cleavage products of gp70(SU) and p27(CA) and gp20(TM) are clearly evident (B) Immunoprecipitation using a mouse anti-gp22 monoclonal antibody raised against SRV-1. The M-PMV precursor $gPr86^{Env}$ is present in the pulse, and cleavage products gp22(TM) and gp20(TM) are present in the chase where gp20(TM) is released into the medium. For PO-1-Lu, only gp22(TM) and gp20(TM) are recognized by this monoclonal antibody.

distinct, an observation in agreement with previous findings (Todaro et al., 1978) The gp22/20(TM) molecules of PO-1-Lu and M-PMV, however, are antigenically cross-reactive, suggesting that these glycoproteins are more conserved (Fig. 4B). The epitopes recognized by the anti-gp70(SU) polyclonal antibody and the anti-gp22/20 monoclonal antibody are not accessible on the PO-1-Lu envelope polyprotein precursor $gPr86^{Env}$. This suggests that the $gPr86^{Env}$ polyprotein precursors for M-PMV and PO-1-Lu are conformationally distinct.

Fig. 2 compares the PO-1-Lu amino acid sequence obtained to that of other retroviruses having related envelope glycoproteins or that belong to the same receptor interference group on human cells. It is clear that sequence similarity was greatest between PO-1-Lu and the exogenous type D retroviruses. Interestingly, certain amino acid motifs were conserved for all of these viruses, namely “DGGGP” and “YHPLA.” When PO-1-Lu was compared only to the exogenous type D retroviruses, these amino acid motifs were slightly longer. “SDGGGPQD”, “YHPLALP”, and a third motif, “CWL”, became apparent. The biological significance of these amino acid motifs remains to be determined. It is not yet known whether they represent conserved regions that contribute to receptor binding.

Due to the degeneracy of the genetic code, the DNA sequence of PO-1-Lu rather than the predicted amino acid sequence was aligned to the entire envelope genes for the viruses shown in Fig. 2 and a phylogenetic tree derived. HIV_HXB2 was used as an outgroup (Fig. 3). The results of the tree reflect the differences noted in the alignment. PO-1-Lu and SRV-6 group together and more closely to SRV-2 than SRV-1 and SRV-3. RD114, BaEV, and SERV represent a distinct but related group of viruses while endogenous SMRV and TvERV/D form one group and the avian SNV and REV form another group from a separate node on the tree. HERV-W, despite sharing a common receptor, is clearly the most divergent.

At the time PO-1-Lu was isolated, M-PMV was the only exogenous type D retrovirus identified. PO-1-Lu was therefore hypothesized as a progenitor to M-PMV only. Our findings, based on available sequence information, support and extend this hypothesis to include the exogenous type D retroviruses of macaques (SRV-1-3) and langur monkeys (SRV-6). The genetic analysis presented here supports the concept that SRV-6 is an exogenous form of PO-1-Lu that has arisen following a zoonosis to a different langur genus. Fig. 2 and 3 imply that PO-1-Lu represents a more immediate progenitor to SRV-2 than SRV-1 and M-PMV (SRV-3). SRV-1 and M-PMV have greater homology to each other than to SRV-2 (Thayer et al., 1987), which is also reflected in Fig 3.

The remarkably close genetic relationship between PO-1-Lu and SRV-6 calls into question the endogenous nature of PO-1-Lu that was originally inferred from hybridization studies of [^{3}H]cDNA derived from in vitro reverse transcription of genomic RNA to langur tissue from both *P. obscurus* and *P. senex* (purple faced langur, now classified as *Trachypithecus vetulus*). PO-1-Lu was also found to exist in multiple copies (20–40 per haploid genome) in *P. obscurus* (now *T. obscurus)* tissue DNA. In addition, the virus exhibited a xenotropic host range and was not pathogenic in its species of origin (Benveniste and Todaro, 1977), features that are shared by many endogenous retroviruses. It is clear that as a truly endogenous retrovirus, PO-1-Lu sequences should be present in all members of the genus *Trachypithecus*. Such a complete analysis has not been performed to

date and would help to confirm the endogenous nature of PO-1-Lu. SRV-6 is unlikely to be an endogenous retrovirus as the infected langur (*Semnopithecus entellus*) produced an antibody response and no treatment of the langur cells (such as iododeoyuridine used for PO-1-Lu) was required to retrieve SRV-6 from infected cells in culture.

The identification of SRV-6 (isolated from *S. entellus*) closely resembling PO-1-Lu (isolated from *T. obscurus*) suggests that zoonoses are actively occurring between primates in Asia. Further transmission to macaques is possible because the exogenous type D retroviruses are naturally transmitted by biting and scratching (trauma), an inherent means by which these primates establish territory and social hierarchy. The Hanuman langur (*S. entellus*) inhabits the same geographical location as macaque monkeys (*Macaca mulatta*) and both can be seen roaming temples and areas of human habitation. This indicates a potential for cross-species transmission between primates and also potentially to humans. Although human cells express receptors, infection of immunocompetent individuals was considered unlikely because of antibody-independent complement-mediated lysis of virions (Welsh et al., 1975, 1976). However, a recent report of low-level persistent infection with SRV-2 in a laboratory worker suggests that immunocompetent persons are susceptible to infection on exposure to infected macaque monkeys (Lerche et al., 2001). It is not yet clear as to whether SRV infection of humans will have any pathogenic effect.

We provide evidence that supports a role for PO-1-Lu as an immediate progenitor to the exogenous type D retroviruses of both langur and macaque monkeys. However, only when the endogenous nature of PO-1-Lu is resolved and complete genomic sequence information is available will the phylogenetic relationship of PO-1-Lu to the known endogenous and exogenous type D retroviruses be formally clarified.

Materials and methods

Cells and viruses

PO-1-Lu and M-PMV were cultivated in human HOS (ATCC: CRL 1543) cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS). Cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Hirt extractions

HOS cells chronically infected with PO-1-Lu and M-PMV respectively were cocultivated with uninfected HOS cells at a ratio of 1:3. Forty-eight to 72 h later, low molecular weight DNA was isolated from the cell cytoplasm by the method of Hirt (1967). This allowed for the isolation of predominantly linear forms of proviral DNA that could be used for Southern blot analysis.

Southern blotting

Hirt extracts corresponding to cytoplasmic DNA including unintegrated proviral DNA were cut with restriction enzymes *Nco*I, *Nde*I, and *Pst*I overnight at 37°C and electrophoresed on a 0.8% agarose gel. The DNA was then transferred to a Genescreen nylon membrane essentially as described by Southern (1975). To generate a genomic M-PMV probe, the full-length infectious molecular clone of M-PMV, pSHRM15, was cut with *Sph*I, which cleaves within the LTR regions of the M-PMV genome, and *Fsp*I, which cleaves within the plasmid backbone that would otherwise be the same size as genomic M-PMV. The M-PMV genomic DNA was gel purified and labelled with fluorescein using the ECL random prime labelling and detection kit according to the manufacturer's instructions (Amersham). The labelled probe was hybridized to the immobilized Hirt extracts (both restriction endonuclease digested and nondigested) at 60°C with the following stringency washes: 1× SSC, 0.1% SDS, and 0.5 × SSC, 0.1% SDS at 60°C for 15 min each. Hybridization of the probe was detected by chemiluminescence.

Polymerase chain reaction (PCR) and sequencing of amplified products

Primers for nested PCR analyses were based on nucleotide sequences available for SRV-2 (M16605) and SRV-6 (AF187057). Nucleotide positions are given for the SRV-2 genome. Outside primers: Con26A (forward) 5′-GCAATGTCCCCACAGTTTTGG-3′ (nucleotide position 6244-6264); Con26D (reverse) 5′-ATGGTAARCATAGCCAGCASTC−3′ (nucleotide positions 6583-6562). Inside primers: Con26B (forward) 5′-GGYCAGCCYRTYTGCTGGAA−3′ (nucleotide positions 6310−6330); Con26C (reverse) 5′CATAGCCAGCASTCRTTGGC-3′ (nucleotide positions 6575–6556). The amplification was performed on a thermocycler (Perkin Elmer, GenAmp PCR System 2400) using a step cycle program starting with a time delay at 94°C for 7 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min for 30 cycles. The nested reaction was similar except that the annealing temperature was 56°C and amplification was carried out for 35 cycles. The PCR products were purified using Q1Aquick PCR purification kit (QIAGEN) following the manufacturers instructions and used directly as templates for DNA sequencing in both forward and reverse orientations using an ABI PRISM 377 DNA Analyse System DNA sequencer (Applied Biosystems, Foster City, CA). Sequencing primers Con26B (forward) and Con26C (reverse) were used under the following conditions: rapid thermal ramp to 96°C, 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min for 25 cycles prior to ethanol

precipitation, electrophoresis, and data collection on the ABI PRISM 377 DNA Analyse System DNA sequencer.

Sequence analysis

Sequence information for PO-1-Lu, compatible in both forward and reverse orientations (submitted to GenBank, accession number AY282754) was translated using the Expasy Translate Tool http://www.expasy.ch/tools/dna.html (Appel et al., 1994) and aligned with the corresponding region of seventeen amino acid sequences derived from viruses that have related envelope genes using the Clustal X(1.8) computer programme. Viral sequences used in this analysis were SRV-6 (AF187057), SRV-2 (L38695; AF126467; AF126468; M16605), M-PMV/6A (M12349), SRV-3 (AF033815), SRV-1 (M11841), baboon endogenous virus (BaEV) strain M7 (D10032); simian endogenous retrovirus (SERV) (U85506; U85505), cat endogenous retrovirus RD114 (X87829), endogenous squirrel monkey retrovirus (SMRV-H) (M23385), reticuloendotheliosis virus (REV) (X01455), spleen necrosis virus (M87666), *T. vulpecula* endogenous retrovirus type D (TvERV/D) (AF284693), and human endogenous retrovirus HERV-W (AF072506).

The amplified DNA sequence of the PO-1-Lu envelope gene within the region encoding gp70 was subject to tree analysis following alignment to the entire envelope genes of the retroviruses described above using Clustal, X(1.8). An unrooted bootstrapped neighbor-joining tree (preserved through 1000 bootstrap replications) was derived from this alignment. The laboratory strain of the lentivirus human immunodeficiency virus HIV_HXB2 (accession number K03455) was used as an outgroup.

Pulse-chase immunoprecipitation assays

PO-1-Lu and M-PMV-infected HOS cells were used for pulse-chase immunoprecipitation assays as described previously (Bradac and Hunter, 1984). Briefly, the cells were seeded at a density of 8×10^5 cells per 60-mm tissue culture dish in duplicate and allowed to settle overnight. The cells were starved in leucine-free DMEM for 1 h followed by pulse labeling with [^{3}H] leucine (100 mCi/dish) for 20 min. The cells were lysed immediately and then immunoprecipitated using either a mouse anti-gp22 monoclonal antibody raised against SRV-1 (Kwang et al., 1987) or a goat anti-MPMV polyclonal antibody (pulse). Parallel cultures were chased in complete medium for 4 h prior to immunoprecipitation. The cell supernatants from the chased dishes were clarified by centrifugation at 2000 rpm for 10 min and also immunoprecipitated. After overnight incubation with antibody, the antibody-antigen complexes were pelleted with Staph A, washed, and finally resuspended in protein lysis buffer. The samples were sonicated and boiled before being loaded onto a 15% polyacrylamide gel and visualized following autoradiography.

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